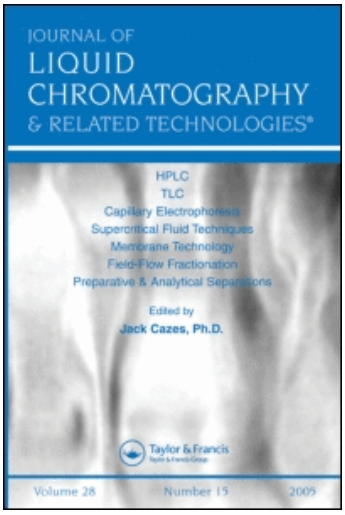


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Homovanillic Acid in Human Plasma Using HPLC with Electrochemical Detection and Automated Solid Phase Extraction

Walther Seiler[a]; Christoph Hiemke[a]
[a] Department of Psychiatry, University of Mainz, Mainz, Germany

To cite this Article Seiler, Walther and Hiemke, Christoph(1993) 'Determination of Homovanillic Acid in Human Plasma Using HPLC with Electrochemical Detection and Automated Solid Phase Extraction', Journal of Liquid Chromatography & Related Technologies, 16: 13, 2813 — 2823
To link to this Article: DOI: 10.1080/10826079308019615
URL: http://dx.doi.org/10.1080/10826079308019615

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(13), 2813-2823 (1993)

DETERMINATION OF HOMOVANILLIC ACID IN HUMAN PLASMA USING HPLC WITH ELECTROCHEMICAL DETECTION AND AUTOMATED SOLID PHASE EXTRACTION

WALTHER SEILER AND CHRISTOPH HIEMKE*

Department of Psychiatry
University of Mainz
Untere Zahlbacher Strasse 8
6500 Mainz, Germany

ABSTRACT

An isocratic HPLC method with electrochemical detection for the quantification of homovanillic acid (HVA) is described. The method included automated solid phase extraction on C_{18}-reversed phase material, followed by separation on a 3-μm Nucleosil 100 C_{18} column (250 mm x 4.6 mm I.D.) with a 100 m*M* citric acid solution (pH 6.6) containing 4% acetonitrile (v/v) as eluent at a flow rate of 0.6 ml/min. Isovanillinic acid served as internal standard. Extractability of both analytes was ca. 80 %. After extraction of 1 ml of plasma, coefficients of variation of replicate analyses were below 10 % in the naturally occuring concentration range.

INTRODUCTION

In man homovanillic acid (HVA, Fig. 1) is the main dopamine metabolite. Hence, changes in the activity of central dopaminergic systems are paralleled by changes in their HVA production (1). Since in man - contrasting with other mammalian species - only a negligible fraction gets conjugated (1), determination of free HVA is therefore frequently used for

* to whom correspondence should be addressed.

both clinical as well as scientific purposes, for example research on psychiatric diseases suggested to be associated with alterations in dopaminergic activity (2). While cerebrospinal fluid (CSF) is not readily accessible and its concentrations of monamine metabolites are very difficult to reliably quantify (3), blood plasma is easily available and monoamine metabolites therein can be determined with high reproducibility (4). Moreover, less than ten percent of centrally formed HVA reach the CSF whereas more than ninety percent enter the blood flow (5). Hence, within limitations (6, 7) changes of HVA concentrations in peripheral plasma are considered to reflect alterations in central dopaminergic activity (5).

For measuring plasma HVA in large sample sizes a method is required which is sensitve and suitable for automation. High performance liquid chromatography (HPLC) with electrochemical detection (ECD) has proved to be a sufficiently sensitive method.

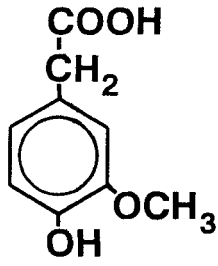
While HPLC-ECD measurement of monoamine metabolites in brain tissue, CSF, or urine can be performed without elaborate sample pretreatment, blood plasma contains a large number of substances appearing in the chromatogram thus requiring a thourough sample purification prior to analysis. A column switching device for analysis of plasma samples as previously described (8) is effective in its purification capabilities and it can be automated but it requires sophisticated instrumentation not available in many laboratories. By contrast, both liquid liquid extraction (9) and protein precipitation (4, 10) are easy to perform. However, these methods are laborious and unsuited for automation.

This paper describes a simple method for the determination of plasma HVA using HPLC with electrochemical detection and automated C_{18} solid phase extraction (SPE) using isovanillic acid (IVA, Fig. 1) as internal standard.

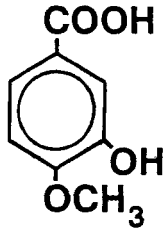
MATERIALS AND METHODS

Chemicals

Homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) and isovanillic acid (3-hydroxy-4-methoxybenzoic acid) were obtained from Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO, USA) respectively. Acetonitrile (Merck, Darmstadt, Germany) and methanol (Merck) were of



HVA



IVA

Figure 1. *Structures of homovanillic acid (HVA) and isovanillic acid (IVA).*

Lichrosolv quality and were used without further purification. The water used in this study was purified and deionized by a Milli-Q water-processing system (Millipore, Eschborn, Germany). All other chemicals were of analytical grade and were purchased from commercial sources.

Standards

Stock solutions of HVA and IVA were prepared by dissolving 10 mg in 10 ml methanol. These solutions could be stored for several weeks in the dark at -20°C without measurable decomposition.

For IVA, the internal standard solutions to be added to the plasma samples were prepared by diluting the stock solution with water to a final concentration of 1 μg/ml. The standard concentrations for the preparation of the HVA calibration curves were obtained by diluting the stock solution with water for appropriate addition to plasma aliquots from the same pool leading to final plasma HVA concentrations of 1, 5, 10, 15, 20, 25, and 30 ng/ml. For each concentration a pool of spiked plasma was prepared and subsequently aliquoted in volumes of ca. 1.1 ml for storage at -20°C.

Chromatography

The chromatographic system comprised a HPLC pump (Bischoff, Leonberg, Germany), an automatic injector, model Promis I (Spark Holland,

Emmen, Netherlands) with a Rheodyne 7010 injection valve and a 100 μl sample loop. For chromatographic separation an analytical column (250 mm x 4.6 mm ID) was used with an integrated precolumn (20 mm x 4.6 mm I.D.) both containing 3-μm Nucleosil 100 C_{18} reversed phase material (cti, Idstein, Germany). The eluent for isocratic elution was prepared by adjusting a 100 m*M* citric acid solution to pH 6.6 with sodium hydroxide pellets. As organic modifier 40 ml acetonitrile was added to 1000 ml aqueous solution. Prior to use the eluent was filtered through a 0.2 μm membrane filter (Millipore). The flow rate was set at 0.6 ml/min. Electrochemical detection was performed using an ESA Coulochem Model 5100 A controller (ESA, Bedford, MA, USA; obtained from Bischoff) with an ESA 5011 dual coulometric detector cell protected by an ESA 0.2 μm graphite filter element. The detector cell's potentials were set at +100 (detector 1) and +600 mV (detector 2) respectively. The signals from detector 2 were recorded and integrated by a D-2000 Merck-Hitachi chromato-integrator (Merck). The entire system operated at room temperature which was set at 20°C.

Extraction

Prior to extraction of the analytes, 1 ml of centrifuged plasma (3000 *g* for 30 min) was mixed with 100 μl of internal standard solution (leading to a final concentration of 100 ng of IVA per millilitre of plasma) and with 1 ml of strongly acidified water (10 ml HCl (30%) ad 500 ml water; pH ca. 0.8) in order to neutralize the acidic functions of HVA and IVA.

The SPE procedure was performed by subjecting the samples to 1-ml (100 mg) C_{18} reversed phase columns (Analytichem Bond Elut, Harbor City, CA, USA; obtained from ict, Frankfurt, Germany) using an automatic sample processor (ASPEC, Gilson, Villiers le Bel, France; obtained from Abimed, Langenfeld, Germany). Prior to sample loading, the columns were sequentially conditioned with 0.5 ml each of methanol and moderatley acidfied water (pH adjusted to ca. 2.0 with HCl (30%)). Loaded columns were washed with 1 ml of 10% methanol in acidified water (pH ca. 2.0). Elution was performed using 1 ml methanol. ASPEC dispension velocities for conditioning, sample loading, washing, and elution were set at 5, 3, 5, and 3 respectively (dimensionless ASPEC-units). The final solvents were evaporated at 40°C in a vortex evaporator (Haake Buchler, Saddle Brook, NJ, USA). For injection into the HPLC system the dried analytes were redissolved in 0.5 ml HPLC eluent.

Extractability was estimated by comparing peak heights of extracted aqueous standards with those obtained after direct injection without extraction.

Quantification

Establishing the HVA peak identity was based on retention characteristics. Calibration of the assay was performed by analysing a series of plasma samples with known amounts of HVA added (final concentrations 1-30 ng/ml). The relative peak heights obtained (HVA peak height / IVA peak height) versus the different HVA amounts added were subjected to least square linear regression analyses. The slopes of these regression lines were used to quantify unknown samples by calculating the quotient [relative HVA peak heights / slope]. Each analytical run contained its own series of calibration samples. During the course of the study reported herein the plasma pool used for preparation of the calibration samples was changed once.

RESULTS

Chromatography

After injection of aqueous standard solutions the chromatographic conditions described above provided a baseline separation of the analytes within less than 20 min (Fig. 2 A). An electrode potential of +600 mV for detection provided the best sensitivity for both analytes (Fig. 3). The detection revealed a signal to noise ratio sufficient to detect 20 pg HVA per inject (Fig. 2 B). Peak identity in chromatograms derived from extracted plasma samples was based on retention characteristics. This identification could be confirmed by inspection of the chromatograms of the calibration samples. Addition of increasing amounts of HVA to aliquots of the same plasma pool led to increasing peak height with no signs of peak asymmetry arising (Fig. 2 D-F). Analytical results obtained from drug free control plasma pools could be repeated with authentic samples from schizophrenic patients receiving neuroleptic medication (Fig. 2 C). Patient's samples did not reveal additional peaks in comparison to chromatograms obtained from healthy volunteers.

Analysis of extracted plasma spiked for the calibration curves revealed a close correlation between HVA amount added and relative HVA peak height

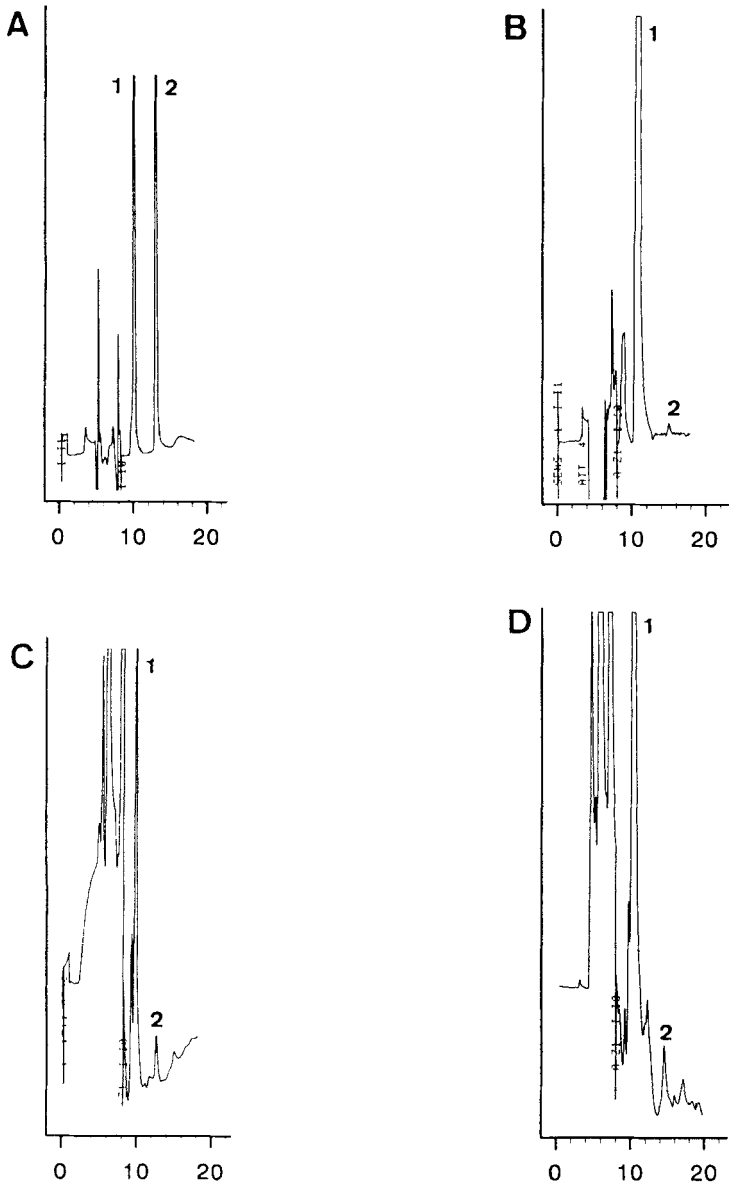


Figure 2. *Typical chromatograms of unextracted standard solutions containing IVA (100 ng/ml) and HVA (A) 100 ng/ml or (B) 0.2 ng/ml; and (D-F) extracted plasma samples spiked with IVA (100 ng/ml) and 0 (D), 15 (E), 25 (F) ng/ml HVA added. (C) Chromatogram of a plasma sample of a schizophrenic patient who was under neuroleptic medication. Peaks labeled as IVA (1) and HVA (2).*

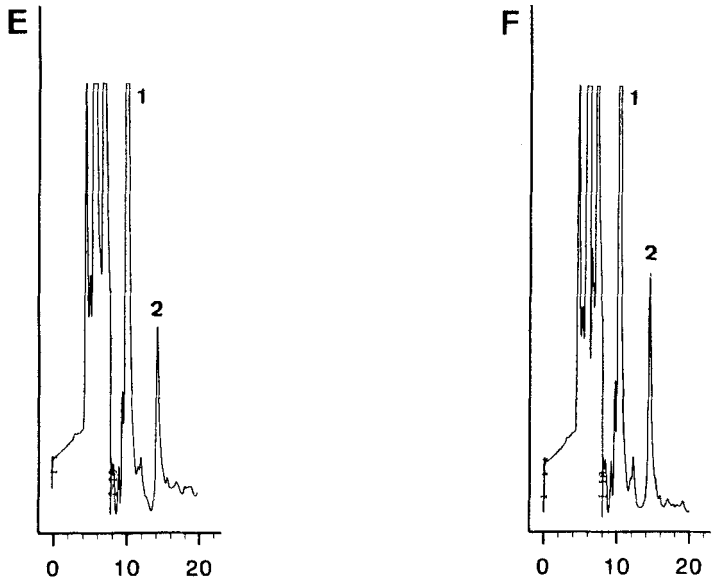


Figure 2 (continued)

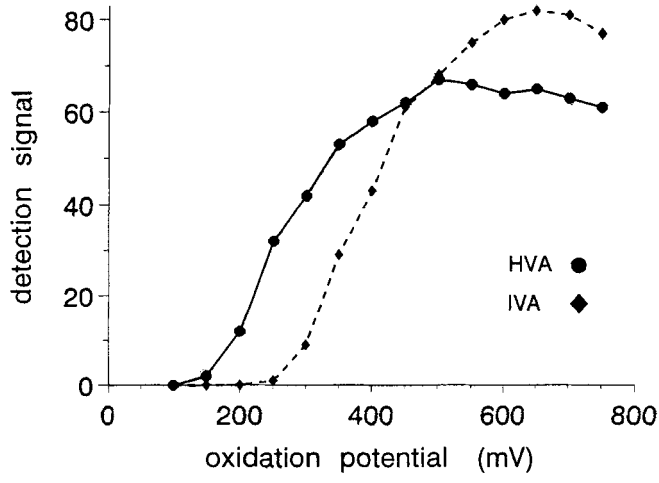


Figure 3. *Voltammograms of homovanillic acid (HVA) and isovanillic acid (IVA) 100 ng/ml each.*

TABLE 1

Assessment of Assay Quality
by Measuring Plasma Samples with Known Amounts of HVA Added

Means ± SD from 15 independent analytical runs all performed on different days. Precolumn, analytical column, detector cell, and plasma pool for the preparation of the calibration samples had been changed during the course of the study. Concentrations of endogeneous HVA in pool plasma ranged between 5.5 and 18.0 ng/ml.

given (ng/ml)	found (ng/ml)	CV (%)	n
1	1.2 ± 0.39	32.5	13
5	5.5 ± 0.68	12.4	13
10	10.1 ± 0.99	9.8	15
15	14.8 ± 0.65	4.4	12
20	19.4 ± 1.18	6.1	11
25	24.5 ± 1.57	6.4	5
30	30.7 ± 1.11	3.6	9

($r = 0.9948 \pm 0.0055$, $n = 15$). The mean (± SD) of the slopes of the calibration curves was found as 0.011 ± 0.0019 ($n = 15$) with no indication of differences between curves resulting from different plasma pools. Data for the estimation of the assay reliability are summarized in table 1.

The detector response was found to decrease with decreasing pH of the HPLC eluent. Chromatography of a directly injected HVA standard solution (100 ng/ml) revealed a peak area of ca. 113000 at pH 2.5, ca. 206000 at pH 4.4, and ca. 400000 at pH 6.6.

Extraction

Extractability on the C_{18} SPE columns was found to be ca. 80 % for both analytes. Recovery was not improved using 90 % methanol or 0.5 *M* NH_3 in methanol as extraction eluent (data not shown). Additional experiments were performed to test an ion exchange sample preparation on strong anion exchange material subsequent to the C_{18} extraction. After loading of the C_{18} eluate on SAX quaternary amine material (Analytichem Bond Elut) recoveries around 100 % were obtained using 1 *M* citric acid

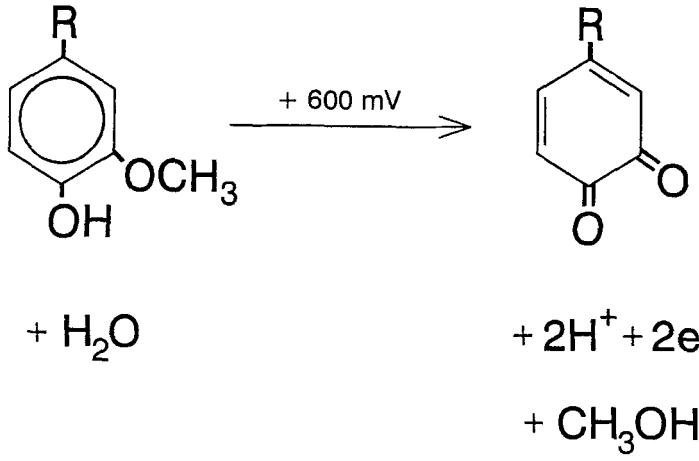


Figure 4. *Reaction scheme of the electrochemical oxidation of a phenyl methoxy molecule giving rise to protons and detectable electrons.*

(adjusted to pH 6.6 with sodium hydroxide pellets) as eluent, which could be directly injected into the HPLC system without evaporation and reconstitution. However, this additional sample preparation step did not improve the purification capability of the single C_{18} extraction (chromatograms not shown).

DISCUSSION

The present system operates with a very simple buffer solution as eluent. There was no obvious need to use complex mixtures as reported by others (2, 5, 11). Moreover, the pH of the HPLC eluent was chosen ca. two pH units above the pK_a values of the analytes (pK_a HVA = 4.4). At pH 6.6 as used here the analytes were separated in a stably dissociated state, thus avoiding partial dissociation which may cause peak asymmetries in the chromatograms. An additional advantage of a more basic HPLC eluent is increased sensitivity since electrochemical oxidation of phenyl methoxy molecules occures by dissociation of a proton (Fig. 4). The lower limit of detection of the present analytical system has not been precisely determined since the sensitivity was sufficient for the determination of human HVA in any plasma sample analyzed so far. The concentrations found here ranged

between 4 and 30 ng/ml which is in agreement with values reported by others (5). Analysis of plasma samples collected from schizophrenic patients receiving neuroleptic medication did not reveal additional peaks thus indicating the method's suitability for measuring plasma HVA in this subject group.

In contrast to other off-line sample preparation procedures SPE can be readily automated without highly sophisticated instrumentation. Moreover, the sample processor may automatically perform even a two-step extraction procedure. Ion exchange chromatography as a second extraction step was tested for additional purification, similar to a two-step SPE of some basic drugs (11). However, anion exchange extraction as a second SPE step did not improve purification results, suggesting that the remaining unidentified peaks in the chromatogram derived from acidic compounds. Nonetheless, the one-step SPE offered sufficient purification as indicated by the reproducibility of measurements of spiked plasma samples (Tab. 1).

In the course of the development of this technique some plasma samples were found to produce unidentified peaks eluting very late (25 min or later). In order to be sure that no interference with chromatograms from the following injection can occur each plasma analysis was routinely followed by subsequent injection of 100 μl methanol. This procedure reliably removed any potential contaminants remaining on the column.

In conclusion, the automated method presented here was found suitable for fast and reliable determination of HVA in plasma samples from normal control subjects as well as from schizophrenic patients. The automation of the otherwise laborious sample preparation procedure was enabled by use of solid phase extraction.

REFERENCES

1 Kopin I.J. *Pharmacol. Rev.* **37**: 333-364 (1985)

2 Mazure C.M., Nelson J.C., Jatlow P.I. & Bowers M.B. *Biol. Psychiatry* **30**: 475-482 (1991)

3 Degrell I. & Nagy E. *Biol. Psychiatry* **27**: 1179-1182 (1990)

4 Chang W.-H., Scheinin M., Burns R.S. & Linnoila M. *Acta Pharmacol. Toxicol.* **53**: 275-279 (1983)

5 Amin F., Davidson M. & Davis K.L. *Schizophr. Bull.* **18**: 123-148 (1992)

6 Lambert G.W., Eisenhofer G., Cox H.S., Horne M., Kalff V., Kelly M., Jennings G.I. & Esler M.D. *Life Sci.* **49**: 1061-1072 (1991)

7 Maas J.W., Contreras S.A., Bowden C.L. & Weintraub S.E. *Life Sci.* **36**: 2163-2170 (1985)

8 Edlund P.O. *J. Pharmaceut. Biomed. Anal.* **4**: 641-652 (1986)

9 Zumàrraga M., Andia I., Bàrcena B., Zamalloa M.I. & Dàvila R. *Clin. Chem.* **33**: 72-75 (1987)

10 Harris P.Q., Bacopoulos N.G. & Brown S.J. *J. Chromatogr.* **309**: 379-384 (1984)

11 Süss S., Seiler W., Hiemke C., Schöllnhammer G., Wetzel H. & Hillert A. *J. Chromatogr.* **565**: 363-373 (1991)

Received: December 10, 1992
Accepted: March 2, 1993